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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(c).

INVENTOR(S)

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Additional inventors are being named on the 0 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Methods and Compositions for Inhibiting the Proliferation of Prostate Cancer Cells

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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[] Application Data Sheet. See 37 CFR 1.76.

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[X] Applicant Claims small entity status. See 37 CFR 1.27.

[X] A check or money order is enclosed to cover the filing fees.

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[] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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Date August 4, 2003

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PROVISIONAL APPLICATION FOR PATENT

under

37 CFR §1.53(c)

TITLE: METHODS AND COMPOSITIONS FOR INHIBITING THE PROLIFERATION OF PROSTATE CANCER CELLS

APPLICANT: CHARLES YOUNG

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METHODS AND COMPOSITIONS FOR INHIBITING THE PROLIFERATION OF PROSTATE CANCER CELLS

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The U.S. Government may have certain rights in this invention pursuant to NIH grant NCI/DK89000 and Army Defense grant DAMID17-98-1-8523.

TECHNICAL FIELD

This invention relates to prostate cancer, and more particularly to methods and compositions for inhibiting the proliferation of prostate cancer cells.

BACKGROUND

The prostate gland is located between the bladder and the rectum and wraps around the urethra. The prostate is composed of glandular tissue that produces a milky fluid and smooth muscles that contract during sex and squeeze this fluid into the urethra where it mixes with other fluid and sperm to form semen. The prostate gland converts testosterone to a more powerful male hormone, dihydrotestosterone, which affects the size of the gland and plays an important role in prostate cancer.

Prostate cancer is a malignant tumor that arises in the prostate gland and can eventually spread through the blood and lymph fluid to other organs, bones, and tissues. Prostate cancer is the most commonly diagnosed cancer in the U.S., and it is the second leading cause of cancer death in American men after non-melanoma skin cancer. Although prostate cancer is just as common in Japan as in the United States, death rates from prostate cancer are significantly lower in Japan. It is unlikely that these differences are all genetic, because Japanese men who migrate to the United States die of prostate cancer with increasing frequency as a function of the number of years they reside in the United States. It is possible that this paradox could be explained, at least in part, by dietary factors.

Benign prostatic hyperplasia (BPH) is a benign enlargement of the prostate gland caused by the growth of both glandular and stromal tissues. Because the prostate enlargement in BPH is affected by testosterone, many men are concerned that it may be related to prostate cancer. A ten-year study, however, found no higher risk for prostate cancer

in men with or that have experienced BPH. BPH develops in the inner zone of the prostate (*i.e.*, predominantly stromal cells), while cancer tends to develop in the outer area (*i.e.*, epidermal cells).

SUMMARY

It is reported herein that the transactivating ability of the androgen receptor was inhibited by the NSAIDs, celecoxib and nimesulide. Accordingly, the invention provides for methods of monitoring the proliferation of cultured prostate cancer cells in the presence of celecoxib and/or nimesulide, methods of treating an individual with prostate cancer or at risk of developing prostate cancer, and methods of reducing the risk of recurrence of prostate cancer in an individual who had previously been treated for prostate cancer. The invention further includes methods of treating an individual with benign prostatic hyperplasia (BPH) as well as methods of screening for compounds that inhibit the proliferation of prostate cancer cells. The invention provides for compositions and articles of manufacture containing celecoxib and/or nimesulide in particular formulations, or celecoxib and/or nimesulide with a second compound that also exerts an effect on the androgen receptor.

In one aspect, the invention provides methods of monitoring the proliferation of cultured prostate cancer cells in the presence of celecoxib and/or nimesulide. Such a method includes contacting the prostate cancer cells with celecoxib and/or nimesulide or a derivative thereof and determining the level of expression and/or transactivating ability of an androgen receptor. Generally, a decrease in expression and/or the transactivating ability of the androgen receptor indicates an inhibitory effect by celecoxib and/or nimesulide on the proliferation of the prostate cancer cells. Representative prostate cancer cell lines include LNCaP cells or LAPC-4 cells.

In another aspect, the invention provides methods of treating an individual with prostate cancer or at risk of developing prostate cancer. Methods of treating an individual with prostate cancer or at risk of developing prostate cancer include identifying an individual with prostate cancer or at risk of developing prostate cancer, administering a dose of celecoxib and/or nimesulide or a derivative thereof to the individual that is effective to inhibit expression and/or the transactivating ability of an androgen receptor, and monitoring the level of expression and/or transactivating ability of the androgen receptor in the individual.

Inhibiting expression and/or the transactivating ability of the androgen receptor inhibits the proliferation of prostate cancer cells, thereby treating the individual. For example, celecoxib and/or nimesulide can be administered to a human, and in an amount of from about 100 mg/kg to about 300 mg/kg. Celecoxib and/or nimesulide can be administered orally, transdermally, intravenously, intraperitoneally, or using an implant.

A method of the invention also can include the step of monitoring the individual for a dose-dependent reduction in prostate-specific antigen (PSA) levels, wherein a dose-dependent reduction in PSA correlates with a dose-dependent decrease in expression of the gene encoding said androgen receptor. Alternatively or additionally, human glandular kallikrein (hK2) levels can be monitored in the individual, wherein a reduction in hK2 correlates with a decrease in expression of the gene encoding the androgen receptor.

In still another aspect, the invention provides for methods of reducing the risk of recurrence of prostate cancer in an individual who previously had been treated for prostate cancer. Such a method includes the step of administering a dose of celecoxib and/or nimesulide or a derivative thereof to the individual that is effective to inhibit expression and/or the transactivating ability of an androgen receptor. The method can further include the step of monitoring expression and/or the transactivating ability of the androgen receptor in the individual. Generally, inhibiting expression and/or the transactivating ability of the androgen receptor inhibits the proliferation of prostate cancer cells, and thereby reduces the risk of recurrence of prostate cancer in the individual. The individual may have previously undergone a radical prostectomy.

In yet another aspect, the invention provides methods of treating an individual with benign prostatic hyperplasia (BPH). This method includes identifying an individual with BPH, and administering a dose of celecoxib and/or nimesulide or a derivative thereof to the individual that is effective to inhibit expression and/or the transactivating ability of an androgen receptor. The method also can include monitoring the level of expression and/or the transactivating ability of the androgen receptor in the individual. Inhibiting expression and/or the transactivating ability of the androgen receptor thereby treats the BPH in the individual.

The invention additionally provides methods of screening for compounds that inhibit the proliferation of prostate cancer cells, including contacting prostate cancer cells with a

compound, and determining the level of expression and/or the transactivating ability of an androgen receptor. The method also can include monitoring expression and/or the transactivating ability of the androgen receptor in the prostate cancer cells. Decreased expression and/or transactivating ability of the androgen receptor in the prostate cancer cells compared to prostate cancer cells not contacted with the compound indicates a compound that inhibits the proliferation of prostate cancer cells. Prostate cancer cells such as LNCaP cells orLAPC-4 cells can be used in this method.

Further, the invention provides compositions that include celecoxib and/or nimesulide or a derivative thereof, one or more compounds that has a particular mechanism of action (*i.e.*, inhibiting expression of a gene encoding an androgen receptor, inhibiting nuclear localization of an androgen receptor, and inhibiting the transactivating ability of an androgen receptor) and a pharmaceutically acceptable carrier. Representative examples of compounds having such particular mechanisms of action include silymarin, silibin, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), quercetin, perillyl alcohol (POH), resveratrol, flufenamic acid, tea polyphenols, and anti-androgen compounds. It is a feature of the invention to provide such a composition in the form of an article of manufacture (*e.g.*, a kit). Such an article of manufacture can include packaging material comprises instructions for using the composition to inhibit expression and/or the transactivating ability of an androgen receptor in an individual.

In another aspect of the invention, there are provided compositions that include celecoxib and/or nimesulide or a derivative thereof and that are formulated for transdermal delivery to the prostate of an individual. Delivery to the prostate typically inhibits expression and/or the transactivating ability of an androgen receptor. In addition, the invention provides compositions that include celecoxib and/or nimesulide or a derivative thereof and that are formulated for implantation near the prostate of an individual. Generally, implantation near the prostate inhibits expression and/or the transactivating ability of an androgen receptor.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and

not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the drawings and detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is a graph showing the effects of NSAIDs on the expression of PSA and hK2 proteins in prostate cancer cells ± 1 nM Mib. LNCaP cells (Panel A) and LAPC-4 cells (Panel B) were treated with or without the indicated concentrations of celecoxib or nimesulide for 7 days. PSA and hK2 values were normalized to growth response measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and expressed as a percentage of that groups treated with Mib only. Error bars indicate the SE of four separate experiments.

Figure 2 is a graph showing LNCaP cells transected with a luciferase reporter plasmid that contains the 6-kb PSA promoter or three copies of ARE or control plasmid (pGL3) and a CMV-β-gal expression vector and treated with NSAIDs ± 1 nm Mib for 24 h. *, $P<0.05$ for PSA promoter and hK2-3ARE promoter. After normalization with β-gal, luciferase activities were expressed as a percentage of that of groups treated with Mib only.

Figure 3A is a graph showing LNCaP cells cotransfected with AR promoter-luciferase reporter (AR-pGL3) or the parental vector (pGL3) and CMV-β-gal and treated with 1 nm Mib and NSAIDs at the indicated concentrations for 24 h. Figure 3B is a graph showing LNCaP cells contransfected with AR promoter (-74/+87)-pGL3, AR promoter (-1380/+577)-pGL3, or the parental vector (pGL3) plus CMV-β-gal and different amounts of c-jun expression vector for 24 h. The resulting activities of both AR-pGL3 were further normalized to β-gal and expressed as a percentage of the AR promoter (-1380/+577) without NSAIDs or c-jun *, $P< 0.05$ for AR promoter (-1380/+577)-pGL3; **, $P<0.05$ for AR promoter (=74/+87)-pGL3.

DETAILED DESCRIPTION

It is reported herein that expression and/or the transactivating activity of the androgen receptor was inhibited by the NSAIDs, celecoxib or nimesulide. Accordingly, the invention provides for methods of monitoring the proliferation of cultured prostate cancer cells in the presence of celecoxib and/or nimesulide, methods of treating an individual with prostate cancer or at risk of developing prostate cancer, and methods of reducing the risk of recurrence of prostate cancer in an individual who had previously been treated for prostate cancer. The invention further includes methods treating an individual with benign prostatic hyperplasia (BPH) as well as methods of screening for compounds that inhibit the proliferation of prostate cancer cells. The invention provides for compositions and articles of manufacture containing celecoxib and/or nimesulide in particular formulations, or celecoxib and/or nimesulide with a second compound that also exerts an effect on the androgen receptor.

It was shown herein that celecoxib and/or nimesulide inhibited androgen-stimulated secretion of both prostate-specific antigen (PSA) and hK2. Expression as well as the transactivating ability of the androgen receptor was diminished by treatment with celecoxib and/or nimesulide. The invention provides a novel aspect of celecoxib and nimesulide in that celecoxib and nimesulide can reduce androgen receptor expression and attenuate androgen receptor-mediated transactivation of prostate cancer-specific genes in androgen-responsive prostate cancer cells. Thus, the invention provides for methods of preventing or treating prostate cancer using celecoxib and/or nimesulide.

The Androgen Receptor and Prostate Cancer

Androgens play an important role in the proliferation, differentiation, maintenance, and function of the prostate. The androgen receptor is the essential mediator for androgen action and is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily. Androgens can enhance androgen receptor protein levels by increasing the half-life, as well as by stimulating the phosphorylation of the androgen receptor. Phosphorylation may affect numerous characteristics of nuclear receptors including ligand binding, nuclear translocation, dimerization, DNA binding, and protein-protein interactions.

Evidence shows that androgens are also involved in the development and progression of prostate cancer. Therefore, the androgen receptor also plays a critical role in the development of prostate cancer, in part due to overstimulation of the receptor by androgens. Prostate cancer also has been attributed to altered transactivation activities of the receptor or to mutations in the androgen receptor that, for example, enable the receptor to respond to non-androgen steroids. The androgen receptor can be expressed in all stages of prostate cancer, and at least one-third of advanced prostate cancers contain amplified androgen receptor genes.

The utilization of androgen deprivation as a treatment for advanced prostate cancer was first demonstrated in 1941 and has become a standard treatment. Based on the morbidity associated with ablation of the adrenal glands, castration alone was the gold standard until the 1980s, when anti-androgen agents, including cyproterone acetate, megestrol acetate, and flutamide, were developed to compete with androgen for binding to the androgen receptor. Many new classes of drugs that interfere with androgen production and function have been identified.

In spite of the apparent regression of tumors by hormone therapy, however, prostate cancer often recurs within 3 years and becomes hormone refractory with a potentially fatal outcome. Many molecular mechanisms have been postulated to be responsible for the development of recurrent hormone-refractory tumors with most involving alterations in the function of the androgen receptor and its complex signaling pathways. The androgen receptor can be activated by a number of growth factors or cytokines in the absence of androgens or by low levels of androgens or other non-androgenic steroid hormones after hormone therapy. That the majority of hormone-refractory cancers still express the androgen-responsive prostate-specific antigen PSA is a protein secreted by the epithelial cells of the prostate gland, including prostate cancer cells. An abnormally high level of PSA is indicative of abnormal prostate cells. (PSA) gene indicates that the androgen receptor signaling pathway is functional.

Nucleic acid sequences encoding androgen receptors have been cloned and sequenced from numerous organisms. Representative organisms and GenBank accession numbers for androgen receptor sequences therefrom include the following: frog (*Xenopus laevis*, U67129), mouse (*Mus musculus*, 109558), rat (*Rattus norvegicus*, 292896), human (*Homo*

sapiens, 105325), rabbit (*Oryctolagus cuniculus* 577829), cow (*Bos taurus*, Z75313, Z75314, Z75315), canary (*Serinus canaria*, 414734), and whiptail lizard (*Cnemidophous uniparens*, 1195596). Additionally, Cancer Genetics Web (www.cancer-genetics.org) contains database entries for wild-type and mutant androgen receptor sequences.

Methods of Monitoring and Inhibiting the Proliferation of Prostate Cancer Cells

The invention provides for methods of monitoring the proliferation of prostate cancer cells. According to the methods of the invention, the proliferation of prostate cancer cells can be monitored by contacting those cells with celecoxib and/or nimesulide and then determining the level of expression and/or the transactivating ability of the androgen receptor using conventional methods (*e.g.*, methods described herein). A decrease in expression and/or the transactivating ability is indicative of an inhibitory effect by celecoxib and/or nimesulide on the proliferation of the prostate cancer cells. Proliferation of prostate cancer cells as used herein refers to an increase in the number of prostate cancer cells (*in vitro* or *in vivo*) over a given period of time (*e.g.*, hours, days, weeks, or months). It is noted that the number of prostate cancer cells is not static and reflects both the number of cells undergoing cell division and the number of cells dying (*e.g.*, by apoptosis). An inhibition of the proliferation of prostate cancer cells can be defined as a decrease in the rate of increase in prostate cancer cell number, a complete loss of prostate cancer cells, or any variation therebetween. With respect to tumors, a decrease in the size of a tumor can be an indication of an inhibition of proliferation.

Prostate cancer cells that can be maintained in culture and are useful in the invention include without limitation LNCaP cells and LAPC-4 cells. The LNCaP cell line is an established androgen-responsive prostate cancer cell line obtained from a lymph node metastasis of a prostate cancer patient. LNCaP cells express the androgen receptor and a number of androgen-inducible genes such as PSA, human glandular kallikrein (hK2), NKX3.1 and ornithine decarboxylase (ODC). The gene encoding the androgen receptor in the LNCaP cell line contains a mutation in its ligand-binding domain, but otherwise is functional. LAPC-4 cells, another androgen responsive prostate cancer cell line suitable for use in the invention, expresses a wild-type androgen receptor. LAPC-4 cells additionally

express PSA and hK2, which are up-regulated in the LAPC-4 cells by androgens. Other prostate cancer cell lines are available and include PC-3 and DU145.

The invention further provides for methods of treating an individual with prostate cancer or at risk of developing prostate cancer. An individual is first identified as having prostate cancer or being at risk for developing prostate cancer and then administered an effective dose of celecoxib and/or nimesulide. Expression as well as the transactivating ability of the androgen receptor can be monitored in the individual to evaluate the effects of celecoxib and/or nimesulide on prostate cancer cells. Generally, an inhibition of expression and/or the transactivating ability of the androgen receptor by celecoxib and/or nimesulide inhibits the proliferation of prostate cancer cells, thereby treating the individual.

Prostate cancer cells can be identified using several criteria. Prostate cancer cells in culture (*e.g.*, LNCaP cells) can be characterized by the response of such cells to androgens or to androgenic agonists or antagonists. Molecular markers, such as increased or decreased expression of androgen-regulated genes or genes involved in prostate cancer (*e.g.*, PSA, hk2, c-jun, ODC, and NKX3.1) also can be used to characterize prostate cancer cells in culture. Prostate cancer *in vivo* can be identified by a digital rectal examination of a patient, or by imaging or scanning techniques (*e.g.*, magnetic resonance imaging (MRI), or prostascint scans). In addition, the degree of cellular differentiation can be evaluated in prostate cancer cells from an individual, typically removed via a biopsy of prostate tissue, using a Gleason score. Further, there are several commercially available diagnostic tests for PSA and PSA-II (*e.g.*, Roche Diagnostics Inc., Indianapolis, IN) to screen individuals for prostate cancer and to monitor individuals undergoing treatment for prostate cancer. Prostate cancer can be staged, for example, using a Partin Table and/or a Partin II Table (see Partin et al., 1994, *Urology*, 43:649-59 and http://www.theraseed.com/gloss.html for more information).

For the purpose of this invention, celecoxib and/or nimesulide can be administered orally, transdermally, intravenously, intraperitoneally, or by implantation. The route of administration typically depends on a variety of factors, such as treatment environment and therapeutic goals. Administration of celecoxib and/or nimesulide can be on a continuous or an intermittent basis. A continuous administration can be, for example, five times a day, once a day, once every other day, once a week, or once a month. In addition, preparations for administration of celecoxib and/or nimesulide can be suitably formulated to give

controlled release of the compound. Preparations for intravenous and intraperitoneal administration can include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents include, without limitation, propylene glycol, polyethylene glycol, vegetable oils, and injectable organic esters. Aqueous carriers include, without limitation, water, as well as alcohol, saline, and buffered solutions. Other additives such as, for example, antimicrobials, anti-oxidants, chelating agents, inert gases, steroids, anti-inflammatory agents, immunosuppressants, vasodilators, vasoconstrictors, and the like may also be present.

Tablets or capsules for oral administration can be prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulfate). Tablets can be coated by methods known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspension, or they can be presented as a dry product for constitution with saline or other suitable liquid vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl- or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for transdermal administration are known in the art. Such transdermal preparations can be in the form of a scrotum patch or a patch for application on the back, abdomen, thighs or buttocks. A transdermal patch typically includes a soft flexible backing (*e.g.*, polyester or polyester/ethylene-vinyl acetate copolymer), a reservoir (in some cases, the compound or composition, *e.g.*, celecoxib and nimesulide, can be deposited as a film on the ethylene-vinyl acetate copolymer or can be combined with, for example, alcohol and a gelling agent such as hydroxypropyl cellulose), and an adhesive backing made out of, for example, polyisobutylene and colloidal silicon dioxide (usually with a removable liner (*e.g.*,

silicone-coated polyester, or fluorocarbon diacrylate) to protect the adhesive until the patch is applied). A transdermal patch also can contain a formulation (*e.g.*, polyisobutylene adhesive) to control the rate of release of the compound or composition.

Implantable devices are known in the art and can be in the form of a pellet or a seed containing or coated with a compound or composition, *e.g.*, celecoxib and/or nimesulide. A pellet or seed can be a metal alloy (*e.g.*, cobalt, or palladium) or an inert plastic or other substance. A device for implantation in or near the prostate can be delivered using a delivery catheter (similar to brachytherapy) and can be deposited in or near the prostate transperineally, transrectally, or transurethrally. A transrectal ultrasound can be used in conjunction with implantation to visualize and image the prostate and the positioning of the implantable device.

According to the invention, an effective dose of celecoxib and/or nimesulide is an amount that inhibits expression and/or the transactivating ability of the androgen receptor, thereby inhibiting the proliferation of prostate cancer cells. Inhibition of expression and/or the transactivating ability of the androgen receptor and the subsequent inhibition of the proliferation of prostate cancer cells can be determined using methods and assays described herein. It is anticipated that an effective dose of celecoxib and/or nimesulide is from about 100 mg of celecoxib and/or nimesulide per kg weight of the individual (mg/kg) to about 300 mg/kg. Toxicity and therapeutic efficacy of different doses of celecoxib and/or nimesulide can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio of LD_{50}/ED_{50}. Doses of celecoxib and/or nimesulide that exhibit high therapeutic indeces are preferred. An effective dose of celecoxib and/or nimesulide can be delivered in a single dose or as multiple doses over a period of time.

The transactivating ability of the androgen receptor can be examined by evaluating the expression of genes whose transcription is regulated by androgen receptor binding. Such genes include PSA, h2k, NKX3.1, and ODC. The amount of transcript and/or protein of such genes in the presence and absence of the compound can be readily determined using art-routine methods such as those described herein. Alternatively, prostate cancer cells in culture

can be made transgenic for one or more androgen-regulated genes and the expression of such transgenes can be evaluated in the presence and absence of a compound.

In addition, the invention provides methods of reducing the risk of recurrence of prostate cancer in an individual that previously had undergone treatment for prostate cancer. Such methods include administering an effective dose of celecoxib and/or nimesulide to the individual such that expression and/or the transactivating ability of the androgen receptor is inhibited. Inhibiting expression and/or the transactivating ability of the androgen receptor inhibits the proliferation, and therefore the recurrence, of prostate cancer cells. Treatments for prostate cancer that an individual might undergo include hormone therapy, chemotherapy, radiation therapy and, oftentimes, a prostatectomy, in which part of all of the prostate gland is removed. A radical prostatectomy includes removal of the entire prostate as well as the seminal vesicles. Due to a high incidence of prostate cancer recurring, even following such treatments (including a radical prostatectomy), methods of the invention provide for administration of celecoxib and nimesulide during or following such treatments. Administration of celecoxib and/or nimesulide may be particularly useful following a radical prostatectomy.

The invention additionally provides for a method of treating an individual with benign prostatic hyperplasia (BPH). Individuals with BPH may present with prostatitis and/or difficulty urinating, and an enlarged prostate due to BPH is typically palpable during a digital rectal exam. Methods of the invention include identifying an individual with BPH, and administering a dose of celecoxib and/or nimesulide or a derivative thereof to said individual effective to inhibit expression and/or the transactivating ability of an androgen receptor. Such an inhibition of androgen receptor expression and of the androgen receptor's transactivating ability reduces the androgen receptor-mediated growth response and thereby treats the individual with BPH.

Methods of Screening Compounds

The invention provides for methods of screening for compounds that inhibit the proliferation of prostate cancer cells by decreasing expression and/or the transactivating ability of the androgen receptor. Screening methods are one of the fundamental tools used in molecular biology for rapid and efficient evaluation of compounds. Screening methods of

the invention include contacting prostate cancer cells with a compound under conditions and for a time sufficient to allow the compound to enter the cell, and determining the level of expression and/or the transactivating ability of the androgen receptor. Generally, decreased expression and/or decreased transactivating ability of the androgen receptor in cells compared to cells not contacted with the compound indicates a compound that inhibits the proliferation of prostate cancer cells. Such compounds can be evaluated using prostate cancer cells in culture, such as LNCaP or LAPC-4 cells, or can be evaluated using a cell-free system.

Methods of evaluation expression and of determining the transactivating ability of the androgen receptor are described above. Expression of a gene encoding an androgen receptor in prostate cancer cells can be examined in the presence and absence of a compound using Northern blot analysis (to evaluate transcription) and/or Western blot analysis (to evaluate translation). Techniques to isolate RNAs and proteins from cells as well as methods of separation (*e.g.*, electrophoretically) are well known and routine in the art. Androgen receptor mRNA can be detected by hybridization with a labeled oligonucleotide probe that is complementary to a portion of the androgen receptor transcript. Androgen receptor proteins can be detected by contacting proteins from a cell with a labeled agent that selectively binds to the androgen receptor protein. Conditions for allowing and detecting hybridization of nucleic acids or binding of antibodies to proteins are well known in the art. Antibodies that have binding affinity to androgen receptor proteins are commercially available (*e.g.*, from Research Diagnostics Inc. (Flanders, NJ) and Alpha Diagnostic International (San Antonio, TX)). The term "label", with regard to an oligonucleotide probe or an antibody is intended to encompass direct labeling of the oligonucleotide or antibody by coupling a detectable substance to the oligonucleotide or antibody, as well as indirect labeling of the oligonucleotide or antibody by reactivity with a detectable substance. Examples of labels and detectable substances are well known in the art. Additional methods to detect androgen receptor mRNA (*e.g.*, RT-PCR or dot blots) or protein (*e.g.*, immunoassays or chromatography) are well known and also practiced routinely in the art.

The ability of the androgen receptor to translocate to the nucleus also can be evaluated in the presence and absence of a compound to determine if the compound inhibits the nuclear localization of the androgen receptor. Nuclei are typically isolated using an

appropriate gradient such as a sucrose gradient, a percol gradient, or the like. The nuclei can be lysed (for example, by exposure to sonication, or ultrasound waves) and androgen receptor protein can be detected using routine methods such as Western blotting. Nuclear translocation also can be examined using, for example, immunocytochemistry to identify androgen receptor protein in the nucleus and/or outside of the nucleus.

In addition, the amount of c-jun protein can be evaluated as an indicator of androgen receptor activity. When overexpressed, c-jun has been shown to inhibit the transactivating ability of the androgen receptor. c-jun is a partner with c-fos in the transcription factor AP-1. Increased evidence suggests that the function of the androgen receptor may be affected by an interaction with AP-1.

Compositions and articles of manufacture

The invention provides compositions that include celecoxib and/or nimesulide or a derivative thereof and at least one other compound selected for its particular mechanism of action on the androgen receptor. The mechanism of action exerted by the other compound(s) can be one or more of the following: inhibition of the expression of a gene encoding an androgen receptor; inhibition of the nuclear localization of an androgen receptor; or inhibition of the transactivating ability of an androgen receptor. Representative compounds exhibiting such mechanisms of action include the following: resveratrol, perillyl alcohol (POH), and omega-3 fatty acids (transactivating ability); silymarin (nuclear localization); flufenamic acid, tea polyphenols (*e.g.*, (-)-epigallocatechin gallate (EGCG)), and quercetin (expression); and numerous anti-androgen compounds (*e.g.*, bicalutamide, flutamide, nilutamide, or cyproterone).

Compositions containing celecoxib and/or nimesulide can be formulated for delivery to the prostate. In one aspect, celecoxib and/or nimesulide is formulated for transdermal delivery to the prostate. In another aspect, compositions containing celecoxib and/or nimesulide can be formulated for implantation in or near the prostate. Delivery of compositions containing celecoxib and/or nimesulide directly to the prostate of an individual inhibits expression and/or the transactivating ability of the androgen receptor. Formulations for administration of celecoxib and/or nimesulide described above and apply as well to the disclosed compositions containing celecoxib and/or nimesulide.

A composition containing celecoxib and/or nimesulide can be in any form provided the composition can be administered to an individual in an amount and for a duration effective to inhibit expression and/or the transactivating ability of the androgen receptor gene, thereby inhibiting the proliferation of prostate cancer cells. Pharmaceutically acceptable carriers include solvents, dispersion media, coatings, antibacterial and anti-fungal agents, isotonic and absorption delaying agents and the like, appropriate to specific routes of administration.

Celecoxib and/or nimesulide compositions of the invention that are effective for inhibiting expression and/or the transactivating ability of the androgen receptor as described herein can be combined with packaging material and sold as a kit (*i.e.*, an article of manufacture). Components and methods for producing articles of manufactures are well known. In addition to a composition containing articles of manufacture can include oligonucleotide probes, antibodies, and/or other useful agents for determining the transactivating ability of the androgen receptor. Instructions describing how the composition can be used for inhibiting expression and/or the transactivating ability of the androgen receptor to thereby inhibit the proliferation of prostate cancer cells can be included in such kits.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1--Cell Culture

Human prostate cancer cell lines LNCaP (American Type Culture Collection, Manassas, VA) and LAPC-4 (kindly provided by Dr. Charles L. Sawyers; Ref. 9) were maintained in RPMI 1640 (Mediatech, Hercules, CA) containing 5% FBS (Biofluids, Rockville, MD) at 37°C and 5% CO_2. To avoid potential interference of existing steroids in FBS, the media were first replaced by serum-free RPMI 1640 for 24 h. Cells were then

cultured in RPMI 1640 with 5% charcoalstripped FBS supplemented with or without 1 nM Mib (New England Nuclear, Boston, MA), a nonmetabolizable, synthetic androgen.

Example 2--Growth Response and PSA and hK2 Levels

Cells were plated in 24-well plates at 2 X 10^4 cells/well. Forty-eight h after plating, cells were treated with celecoxib or nimesulide (LKT Lab, St. Paul, MN) and other NSAIDs as shown in Table 1 at different doses in the presence or absence of Mib. MTS assay (Promega, Madison, WI) was performed to determine cell proliferation 6 days after the treatment. To measure secreted PSA and hK2 levels, 400 µl of spent medium from cells treated for 6 days were collected. PSA and hK2 proteins levels were determined using specific immunoassays (Mayo Immunochemical Core Facility). These measurements were used to the calculate 50% inhibitory concentration (IC50) of each of the NSAIDs.

Table 1 Effects of selected NSAIDs on growth responses and expression of androgen-regulated genes in androgen-responsive human prostate cancer cell lines

		LNCaP (IC_{50})[a]			LAPC-4(IC_{50})[a]		
NSAIDs	Selective inhibitor to	Growth	PSA	HK2	Growth	PSA	hK2
Asprin	COX-1 and -2	>1000	>1000	>1000	>1000	>1000	>1000
Ibuprofen	COX-1 and –2	>1000	783.3	860	740	870	803
Meloxicam	COX 2	193.1	300	377	92	>300	>300
Ketoprofen	COX –1 and –2	>300	>300	>300	>300	>300	>300
Flurbiprofen	Cox-1 and – 210476.5	>300	206	281	347	234	221
Nimesulide	COX>300>300-2253	38.2	27	23	104	76.5	61
Sulindac	COX44.5-1 and –2 N/D[c]	>300	>300	>300	>300	>300	>300
Sulindac sulfone	*b*	>3001	206	97.6	253	193.8	>300
Celecoxib	COX-2	32.6	20	29.6	44.5	28.7	43.7
Fenoprofen	COX-1 and –2	>300	210.4	228.6	N/D[c]	N/D	N/D
Indomethacin	COX-1 and -2	>300	224	272	170	203	207

Example 3--Western Blot Analysis

Cells were seeded at 1 X 10^5 cells/ plate in 100 mm dishes. Cells grown in log phase were co-treated with 1 nM Mib and different concentrations of celecoxib or nimesulide for 15 or 24 h. The cells were collected by centrifugation and washed with cold PBS. Cell lysates were prepared in radioimmunoprecipitation assay buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS plus freshly added protease inhibitors, 100 μg/ml phenylmethylsulfonyl fluoride, 30 μl/ml aprotinin, and 1 mM sodium orthovanadate) and used for Western blot analysis. The sample filters were immunoblotted with c-Jun, phospho-c-Jun (Cell Signaling, Beverly, MA), AR (PharMingen, San Diego, CA), FKBP51 (a gift from Dr. D. O. Toft; Mayo Clinic) specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ).

Example 4--Transfections and Transcriptional Reporter Assays

LNCaP cells were plated into 60-mm dishes. Cells at 50–70% confluence were transfected with the appropriate constructs [6-kb PSA promoter-pGL3, AR promoter (-74/+87)-pGL3, AR promoter (-1380/+577)-pGL3, hK2 3xARE-SV40 minimal promoter pGL3, or empty pGL3 vectors] by using the method described previously (Ren et al., 2000, *Oncogene*, 19:1924-32). Twenty-four h after transfection, cells were treated with celecoxib or nimesulide in combination with Mib or ethanol. Whole cell lysate was prepared for luciferase assay according to the manufacturer's instructions (Promega). CMV-β-gal expression vector was also cotransfected for normalization of transfection efficiency. Each transfection was done three times, and SDs were calculated.

Example 5--Statistics

The data were analyzed by Student's *t* test. $P < 0.05$ was accepted as the level of significance.

Example 6--Celecoxib and Nimesulide Inhibited the Expression of Androgen Up-Regulated Genes

The effects of several NSAIDs on inhibition of androgen action and growth in prostate cancer cells was examined. Using PSA and hK2, two well-established AR target genes, as markers, the effects of a panel of 11 NSAIDs was tested on androgen action in two androgen-responsive human prostate cancer cell lines, LNCaP and LAPC-4, respectively. Among the NSAIDs tested, COX-2-specific inhibitors seem to have a higher potency than other NSAIDs in inhibiting androgen action. Celecoxib and nimesulide showed the lowest IC_{50} concentrations in both cell lines (Table 1). Because of their highest potency on inhibition of cell growth and androgen function, celecoxib and nimesulide were chosen for additional studies. Figure 1 illustrates that expression of PSA and hK2 was suppressed by celecoxib and nimesulide in a dose-dependent manner in the two cell lines. Significant inhibitory activity was observed for celecoxib at a concentration of 10 μM for both PSA and hK2. Nimesulide at 10 μM resulted in a similar inhibition of PSA, although a higher concentration was required to achieve significant down-regulation in LAPC-4 cells. Recently, it was discovered that FKBP51, an immunophilin, is up-regulated by androgens. Similarly, it was found in the experiments described herein that androgen-up-regulated FKBP51 protein expression was alleviated by celecoxib and nimesulide treatment as measured by Western analysis using specific antibody. These results suggest these NSAIDs are potent inhibitors of AR-induced gene expression.

Example 7--Celecoxib and Nimesulide Inhibited AR-induced Gene Expression and AR Promoter Activity at the Transcription Level

To test whether celecoxib and nimesulide can directly repress the promoters of AR-dependent genes, reporter assays were performed using a PSA promoter-luciferase construct. As can be seen in Figure 2, celecoxib and nimesulide significantly reduced the androgen-induced PSA promoter activity at a concentration as low as 25 μM. Because the AR binds directly to the ARE of target genes for androgen action, another reporter construct containing three tandem repeats of ARE derived from the hK2 promoter (pGL3 hK2 3xARE SV40 minimal; Ref. 12) also was tested. The result (Figure 2) demonstrated that both celecoxib

and nimesulide significantly reduced AR/ARE-mediated gene expression ($P < 0.05$). Thus, celecoxib and nimesulide acted as potent inhibitors of AR-mediated gene transcription.

To determine whether celecoxib and nimesulide may directly affect the transcriptional activity of the AR gene, transcriptional reporter assay was performed in LNCaP cells using a luciferase reporter plasmid containing the AR promoter (-1380/+577). Compared with control vector alone, cells transfected with the AR promoter revealed significantly higher luciferase activities, as expected (Figure 3A). However, celecoxib and nimesulide, at the concentrations used, repressed the transcription activities of the promoter (Figure 3A). Furthermore, Western analysis using AR-specific antibody indicated that AR protein expression was reduced by celecoxib and nimesulide at concentrations used in the transfections. Taken together, these results suggest that celecoxib and nimesulide are potent inhibitors of AR function at least partially through downregulation of AR expression.

Example 8--Enhanced Expression and Phosphorylation of c-Jun by Celecoxib and Nimesulide in LNCaP Cells

To further dissect the molecular mechanisms underlying NSAID-mediated inhibition of AR function, the expression of c-Jun in celecoxib- and nimesulide-treated LNCaP cells was examined by Western blot analysis. Androgen-induced PSA promoter activity was previously shown to be inhibited in a dose dependent manner by cotransfection with c-Jun expression plasmid. It was hypothesized, therefore, that c-Jun may potentially be involved in NSAID-mediated inhibition of AR. Results demonstrated that c-Jun protein was strongly induced by celecoxib and nimesulide at 24 h of treatment. Several previous studies have shown that the transactivation functions of the AR as well as other steroid receptors can be affected by c-Jun. Therefore, the results obtained herein strongly suggest that overexpressed c-Jun induced by celecoxib and nimesulide could interfere with AR-mediated up-regulation of PSA and hK2. It is noted that celecoxib at a relatively low concentration of 25 μM may not have an observable inhibitory effect on AR protein expression, but low concentrations of the NSAIDs could still increase c-Jun protein expression and subsequently reduced the function of the AR, as evident in the transfections shown in Figure 2.

c-Jun is usually a short-lived protein, and it can be induced by many extracellular stimuli. In most cases, the induction is transient at early time of stimulation. However, the

results described herein show that c-Jun protein levels were elevated with 15 and 24 h of treatments, implying that the NSAIDs induced a prolonged overexpression of c-Jun.

Example 9--Overexpression of c-Jun Inhibited the AR Promoter

To determine whether overexpression of c-Jun can affect the expression of the AR gene, c-Jun expression construct was cotransfected with the two AR promoter reporter plasmids, AR promoter (-1380/+577)-pGL3 and AR promoter (-77/+84)-pGL3, respectively, in LNCaP cells. The result shown in Fig. 3B suggests that overexpression of c-Jun significantly inhibited the activity of both tested AR promoters.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

ABSTRACT

The invention provides for methods of monitoring the proliferation of cultured prostate cancer cells in the presence of celecoxib and/or nimesulide, methods of treating an individual with prostate cancer or at risk of developing prostate cancer, and methods of reducing the risk of recurrence of prostate cancer in an individual who had previously been treated for prostate cancer. Methods of the invention further include treating an individual with benign prostatic hyperplasia (BPH) with celecoxib and/or nimesulide as well as methods of screening for compounds that inhibit the proliferation of prostate cancer cells. The invention provides for compositions and articles of manufacture containing celecoxib and/or nimesulide in particular formulations, and celecoxib and/or nimesulide with a second compound that also exerts an effect on the androgen receptor.

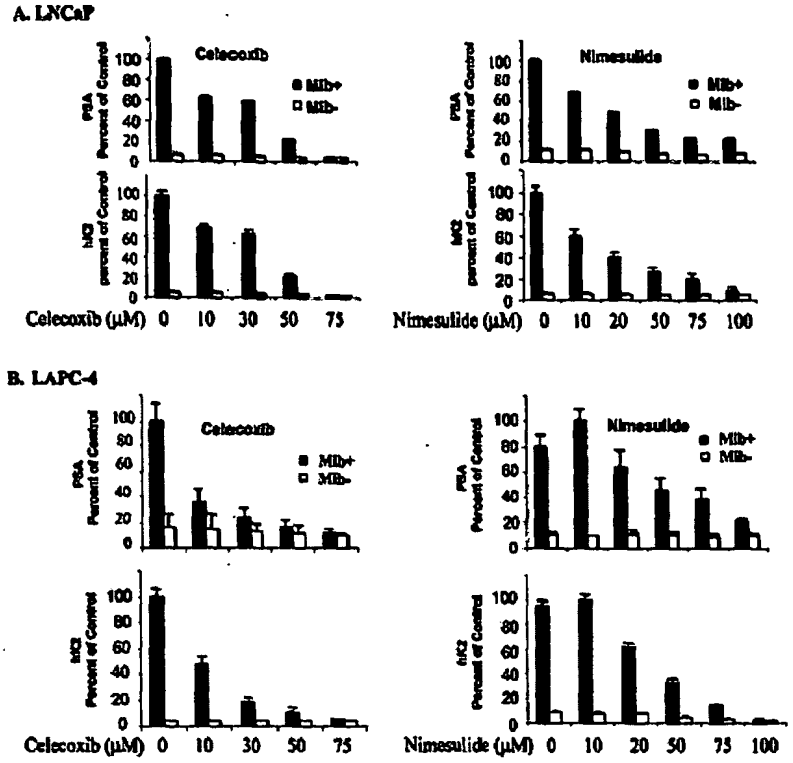
A. LNCaP
Celecoxib
Nimesulide
Mib+
Mib-
PSA Percent of Control
hK2 percent of Control
Celecoxib (μM) 0 10 30 50 75
Nimesulide (μM) 0 10 20 50 75 100
B. LAPC-4
Celecoxib
Nimesulide
Mib+
Mib-
PSA Percent of Control
hK2 Percent of Control
Celecoxib (μM) 0 10 30 50 75
Nimesulide (μM) 0 10 20 50 75 100

Figure 1

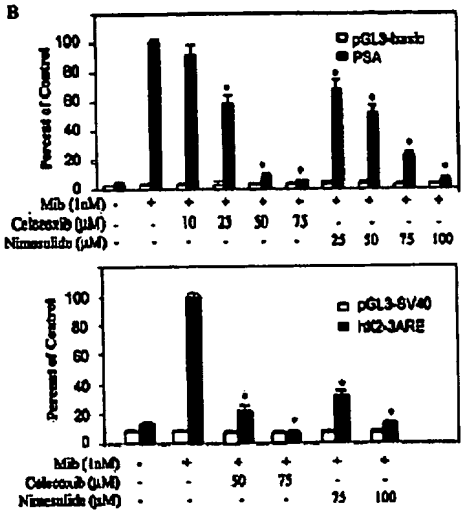
B
Percent of Control
pGL3-basic
PSA
Mib (1nM)
Celecoxib (μM)
Nimesulide (μM)
pGL3-SV40
hGC-3ARE

Figure 2

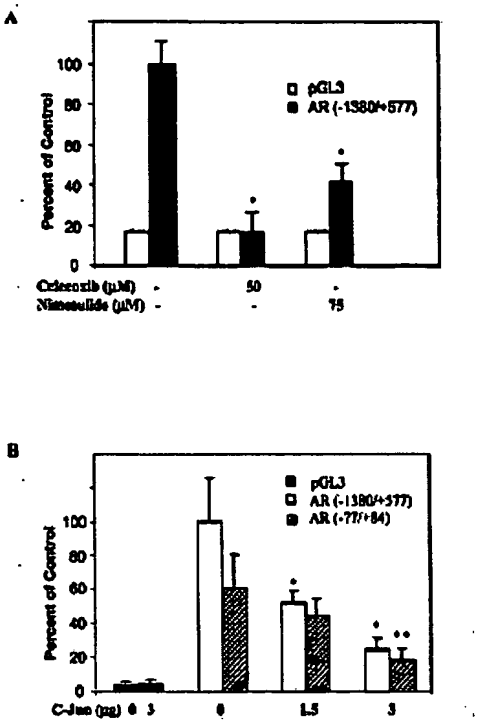
A
Percent of Control
100
80
60
40
20
0
pGL3
AR (-1380/+577)
Celecoxib (μM) -
50
Nimesulide (μM) -
75
B
Percent of Control
100
80
60
40
20
0
pGL3
AR (-1380/+377)
AR (-77/+84)
C-Jun (pg) 0
3
0
1.5
3

Figure 3

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